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Round Robin test on bio-imaging transfer standard for 3D optical profilers

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ABSTRACT

A stair case height Bio-Transfer-Standard (BTS), developed and produced at the University of Helsinki (UH), was measured in two laboratories. The Round Robin test aims to determine whether BTS works with different optical profilers in different laboratories. First the artefact was measured at UH using a custom-built Scanning White Light Interferometer. Then BTS was measured at Sensofar-Tech, S.L. using an S-neox-type interferometer working either in Phase Shifting Interferometry mode or in Imaging Confocal Microscopy mode. To remove the influence of system calibration, a method featuring sample shifting and measurement subtraction was used. The BTS features eight lipid bilayer steps that each are 4.6 ± 0.1 nm tall on average. All 30 measurements done by four different operators at the two laboratories agree to within 0.1 nm which agrees with theoretical estimates and with measurements done using a surface plasmon resonance technique. The Round Robin results show the applicability of the newly developed bio-imaging transfer standard for calibrating 3D optical profilers.

Keywords: Bio transfer standard, Optical Profiler, Coherence Scanning Interferometry, Confocal, Phase Shifting Interferometry.

1. INTRODUCTION

Bio-imaging, especially label-free bio-imaging, is globally large and is rapidly growing both academically and commercially. The main trends are 3D imaging, super-resolution imaging (Nobel Prize 2013), high-throughput imaging (currently hundreds of samples per microscope per day), label-free imaging (no fluorophores or stains), fresh-sample imaging (little or no sample preparation), and quantitative (metrologically traceable) imaging. For the last one there is a need for a soft transfer standard. Without traceability, measurement results are not repeatable and cannot be compared across laboratories, or even compared to results from the same laboratory obtained at different times or with different instruments. The high resolution and good repeatability of modern microscopes may give an illusion of high accuracy. Frequent calibration, instrument stability, and high resolution are all needed to have reliable and accurate measurements.

Today, measurement instruments are classified as contact and non-contact techniques. Most of the non-contact instruments provide areal measurements and they are calibrated following the procedures described in old ISO normatives for contact instruments. Existing calibration specimens are made on hard substrates, such as metal, glass or silicon. Most of these calibration standards are above 1 micron step height, and only some of them have less than 50 nm height. There have been several attempts to manufacture a commercial step height standard with less than 10 nm height. Several techniques have been proposed to do so, such as crystalline surfaces, nano-origami, or self-assembled structures[1].

A single step height standard containing only one traceable height value is useful to calibrate the amplification coefficient of the instrument scanner, responsible at the end of the accuracy of the measurement. Successive measurements close to the calibrated value will have traceability, but this will not ensure that heights above or below the calibrated one will keep good accuracy. To ensure that, a set of several step height standards covering the desired height ranges that wants to be measured need to be used for the calibration of the amplification coefficient and linearity.

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To meet these needs a stair case height Bio-Transfer-Standard (BTS) was developed and produced at the University of Helsinki (UH), Finland. To study the performance of the BTS a study – a Round Robin test – was performed. First the artefact was measured at UH using a custom-built Scanning White Light Interferometer[2]. Then the BTS was measured at Sensofar-Tech, S.L. using an S-neox working either in Phase Shifting Interferometry[3] mode or in Imaging Confocal Microscopy[4] mode.

2. METHODS

All metrological applications rely on a calibration standard: a sample with known dimensions. The standard permits the measuring system (instrument) to be calibrated and its accuracy to be verified. To be scientifically valid, the material and dimensions of the transfer standard (TS) should closely match the properties of the actual sample that the system measures. Current transfer standards are made of metal, silicon, and other hard and technically oriented materials. The refractive index and surface roughness of such standards are very different from those of bio-samples and therefore do not offer a valid calibration for applications in the field of bio-imaging.

Step height measurement on cross profiles follow the ISO 5436-1 method. The ISO procedure is described for step height groove types, with a precisely localized step and two side locations for height referencing. The ISO procedure states to measure the width of the step (w) and avoid 1/3 of the width to remove any effect of the step height walls (Fig. 1).

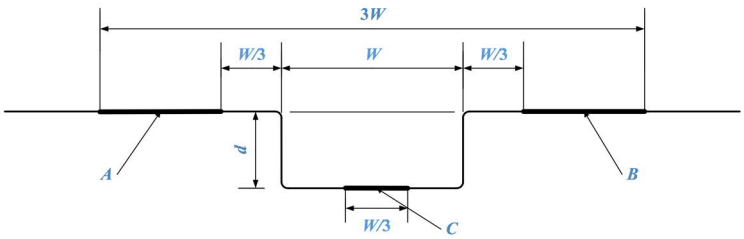

Figure 1. Step height measurement procedure described in the ISO 5436-1.

The above method is currently used on 3D measuring instruments, but it does not use the benefit of areal information, but only 2D information on a cross profile. Currently the ISO TC213 WG 16 is working on a draft of the ISO 25178-700 to describe the calibration specimens that will be intended to use for the calibration of the amplification coefficient and linearity.

2.1 Bio transfer standard

Nature is 3D, and it can't be truly understood through 2D images. Existing technologies for manufacturing 3D TS rely on mechanical processes, most of the times with manually operated machines. To characterize bio-samples in a 3D and quantitative manner measurements should be calibrated both in the out-of-plane direction and in the in-plane direction. Currently existing nm-size calibration standards are unsuitable for use with bio-samples, because of phase change in light upon transmission or reflection. For true imaging a TS made by a material optically similar to the sample under investigation is necessary. To address the need for having true 3D images we introduce a TS whose dimensions are 'guaranteed' by natural self-assembly.

The BTS is based on applying Langmuir-Blodgett films (LBF) of a certain material (e.g. stearic acid) onto a desired surface (e.g. mica or glass microscopy slide) to form a flight of steps. The nature of the LBF makes every single layer equally thick, only a few nanometers tall. Partly overlapping placement (horizontal offset) of these films produces a step structure suitable for both off-line instrument calibration and for online and in-view calibration purposes (Fig. 2).

The BTS was manufactured by the Langmuir-Blodgett technique by using a KSV Minitrough (KSV Instruments, Helsinki, Finland). Briefly, a monolayer of stearic acid was first spread onto a sub-phase containing 50 μM Uranylacetate (UAc) and compressed at 10 mm/min to a surface pressure of 45 mN/m. Then, 8 bilayers of the stearic acid monolayer were

transferred onto a glass microscopy slide. This was achieved by intermittently immersing and withdrawing the slide into the subphase through the stearic acid monolayer 16 times at a speed of 2 mm/min, while keeping the surface pressure constant at 45 mN/min during the entire deposition process. The flight of the steps was formed during the deposition process at the three-point contact line between the microscope slide, air, and the monolayer covered subphase. The distance between the flight of steps was realized by immersing the slide to a different depth during each bilayer deposition cycle. The thickness of similarly prepared stearic acid monolayers have been determined with a surface plasmon resonance technique to be between 2.24 – 2.48 nm, which corresponds to a bilayer thickness between 4.48 – 4.96 nm[5].

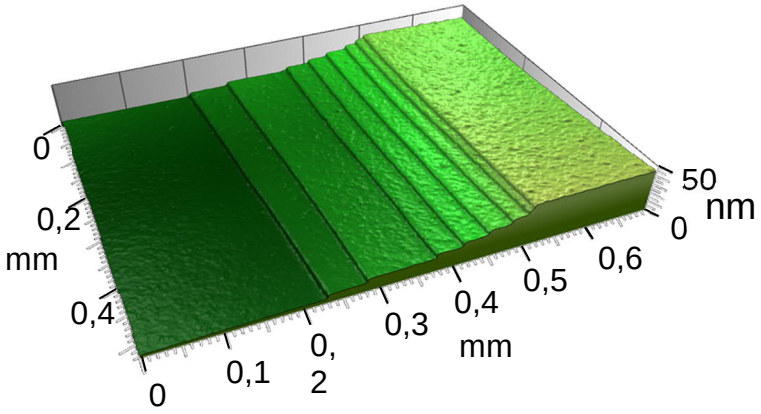

Figure 2. Langmuir-Blodgett films (LBF) based Bio-Transfer-Standard. Partly overlapping placement (horizontal offset) of the LBFs produces a step structure suitable for both off-line instrument calibration and for online and in-view calibration purposes.

2.2 Optical 3D measurements systems

Three dimensional measurements of surfaces is an accepted technique in many fields. There are many different optical techniques that provide height sensitivity with a few nm of resolution. In general, optical methods are either single point or imaging detectors. Single point detectors measure the height within a projected spot, and the 3D reconstruction is done by scanning the sample or the detector in a raster scan. Laser confocal, chromatic confocal, triangulation, and dynamic focusing are common techniques. These sensors are fast for single profile acquisitions, but slow if the intention is to measure areal topographies. Moreover, the height resolution is typically limited to few nm. Imaging sensors employ a camera to simultaneously gather height information in each pixel. The most common 3D optical profilometers are focus variation, interferometric, and confocal microscopes.

Focus variation relies on the shape-from-focus technique, in which an algorithm detects high frequency details in the surface under inspection. Once the surface is out of focus, the details are blurred, and the high frequency details are lost. This kind of microscope is useful for rough surfaces, but it is limited when the surface is optically smooth, e.g. with glass, semiconductors, and polymers.

Interferometers resolve height deviations below 1 nm. If the interferometer employs a broadband spectrum light source, such as a white light LED, the interferometer is called Coherence Scanning Interferometer (CSI). The recorded signal along the optical axis is close to sinusoidal shape with its amplitude modulated by a Gaussian function with narrow width, on the order of the coherence length of the light source. The surface height is retrieved from the position of the maximum of such amplitude. The instrument noise in a CSI system is close to 1 nm. If the interferometer features a narrow band light source, such as a monochromatic LED, the interferometer is called a Phase Shifting Interferometer (PSI). In this case, the signal recorded along the optical axis has a long amplitude modulation due to the large coherence length of the light source. In PSI systems, the phase offset for each pixel is measured resulting in a phase map of the surface. This map which can be converted into a height map with the use of the mean wavelength of the light source. PSI systems achieve low instrument noise, even as low as 0.05 nm.

Confocal systems are a trade-off between interferometers and focus variation, and are therefore the most versatile instruments in terms of application range. The optical and mechanical arrangement of a confocal microscope is complex, but it suppresses the signal that falls outside the depth of focus of the objective. Thus, the recorded signal along the optical

axis resembles a Gaussian function, where the position of its maximum corresponds to the location of the surface. The instrument noise, which depends on the numerical aperture (*NA*) of the objective, can reach 1 nm for objectives with a *NA* = 0.95.

2.3 Confocal and PSI

Several arrangements provide a microscope the capability of making confocal images. According to ISO25178-607 there are three main technologies: laser-scan, disc scan, and microdisplay scan confocal microscopes. A laser scan confocal microscope uses a laser light source to illuminate a pinhole that is projected onto the surface under inspection. The light reflected or scattered from the surface is imaged back into a second pinhole, the confocal aperture, which filters out the light that is reflected outside the depth of focus of the objective. The beam is raster scanned to cover a desired field of view. In a disc scanning confocal microscope, a disc with a pattern of opaque and transparent regions, usually a large number of pinholes or slits, is imaged onto the surface. A light source illuminates the required area on the disc needed to fill the desired field of view. The light reflected from the surface is imaged back through the same pattern, providing the light rejection. The pattern is imaged onto a camera where a confocal image is recorded. In both confocal arrangements, the illumination and observation light path does not allow one to record a bright field image. The instrument is therefore incompatible with an interferometer. To solve this problem instruments that require a bright field image for sample manipulation feature a second light source and a beam splitter before the pupil of the microscope's objective to allow bright field illumination and observation through a dedicated camera. In contrast, a microdisplay scan confocal microscope (Fig. 3) can use the same illumination and observation optical path to acquire a confocal, and interferential images[4].

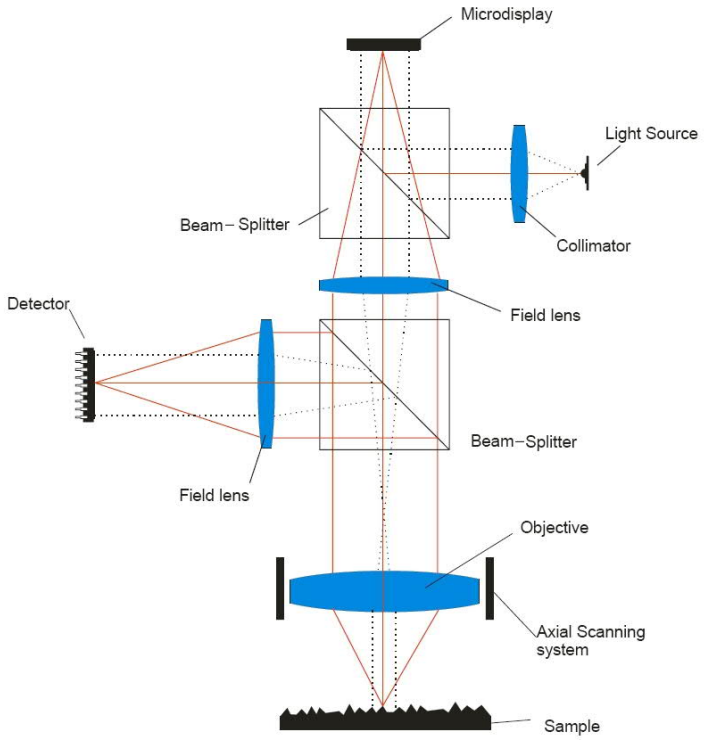

Figure 3. Optical setup of a Microdisplay Scan Confocal microscope. This setup allows for 3D acquisition of confocal and interferometric measurements.

A light source is collimated and directed onto a microdisplay, which is located on the field diaphragm position of a microscope's objective. The microdisplay is reflective, and it can be based on e.g. FLCoS (ferroelectric liquid crystal on silicon) or DMD (digital micromirror device). Each pixel in the microdisplay is imaged onto the surface, and by the use of a field lens, the surface and the microdisplay are simultaneously imaged onto a camera. To have the same performance as a laser scan microscope, a single pixel of the microdisplay is switched on (reflective), while all the other are switched off. A single point of the surface is illuminated, and the corresponding single pixel of the camera records the signal. Optical sectioning light rejection is achieved by recording the signal of a single pixel of the camera, which behaves as a confocal aperture pinhole and detector simultaneously. A raster scan of all the pixels of the microdisplay creates a confocal image in the same way as in a laser scanning system. Parallel illumination and signal recording is achieved by switching ON a set of equally distributed pixels, slits or other patterns that confines the amount of illuminated regions.

To acquire an interference signal, a Mirau type objective is used in combination with a white light or monochromatic LED, and all pixels in the microdisplay are switched ON. By recording a single image of the camera one recovers an interference image, which in combination with the vertical scanner allows CSI or PSI measurements.

2.4 Low noise measurements of PSI and Confocal

When measuring small step heights using Phase Shifting Interferometry or Confocal devices, maximum attention should be paid on instrument noise. In confocal measurements, the instrument noise depends on the *NA* of the objective. The left side in Figure 4 shows the axial response of a confocal microscope for diferent *NA*. High numerical aperture objectives are available with magnifications ranging from 50X to 150X. Figure 4 (right) shows the instrument noise as a function of *NA*. For low magnification objectives, with low *NA*, some hundreds of nm noise level is present, while 1 nm noise level is reached with the highest magnifications. Measurements of the Bio-Transfer Standards in this paper were done with a 0.95 = *NA* objective featuring 50X magnification.

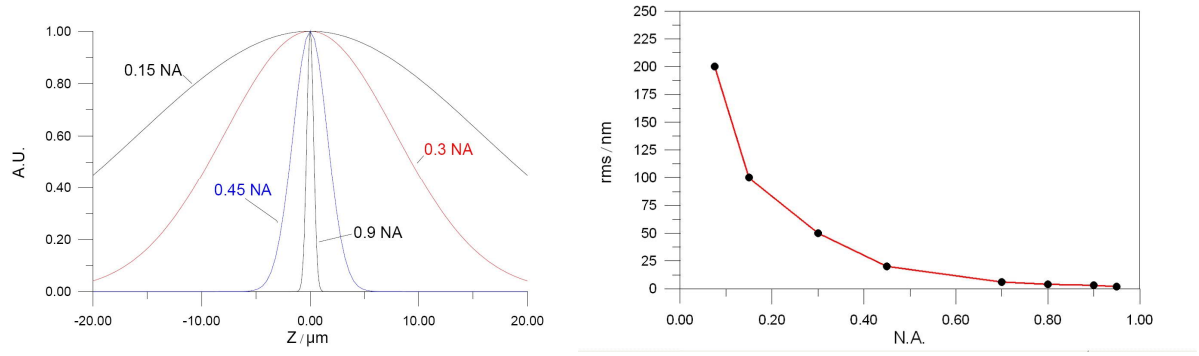

Figure 4. Left: measured axial response for objectives with different numerical apertures. Right: instrument noise as a function of numerical aperture.

In addition to instrument noise, confocal microscopes exhibit residual flatness error. This error is present as a low frequency component superimposed onto the surface, which originates from the residual field curvature of the microscope's objective. This small error is evident as a smooth varying topography. This artifact is determined during calibration and it is subtracted from each consequent measurement, but slight differences between the actual measure and this calibration are still present.

Phase Shifting Interferometry can achieve sub-Ångström instrument noise. Nevertheless, when measuring with a Mirau or Michaelson type interferometer, the instrument noise is limited to the value of the roughness of the reference mirror. Figure 5 shows the topography of the reference mirror, which has its own roughness (Sq), typically between 0.3 and 0.7 nm.

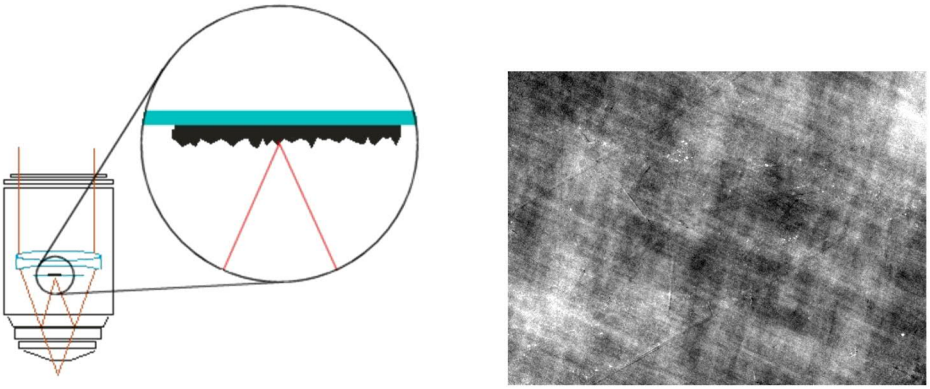

Figure 5. Mirau type interferometer (left) showing the topography of the reference mirror. Right: the topography of 0.3 nm Sq roughness of a reference mirror.

To supress the influence of the reference mirror, a precise measurement of its surface is done and the result is subtracted from subsequent measures. This reference topography is the average topography measured at many places, typically more than 10, on a reference SiC mirror. A small lateral shift is done for every topography within the series of topographies to ensure that the texture of the SiC mirror is averaged and only those components which correspond to the reference mirror are left. Figure 5 (right) shows a topography of a 20X Mirau objective that was determined with this method.

To achieve very low instrument noise in Phase Shifting Interferometry after suppression of the reference mirror topography, the system has to be very well vibration isolated, and averaging techniques have to be used. Typical values after averaging more than 16 images are 0.05 nm of Sq. Nevertheless, any small relative tilt between the reference topography and the actual measurement creates residual flatness errors that appear as low frequency components.

2.5 Lateral shift techniques

Both, confocal and PSI techniques require precise calibration and measurement conditions to achieve measurements with low residual flatness error. Figure 6 shows a BTS measurement, where nm-level step heights are visible. Low frequency components of less than 0.2 nm height are also visible (the colour scale has been enhanced to provide a clear view of the residual flatness error).

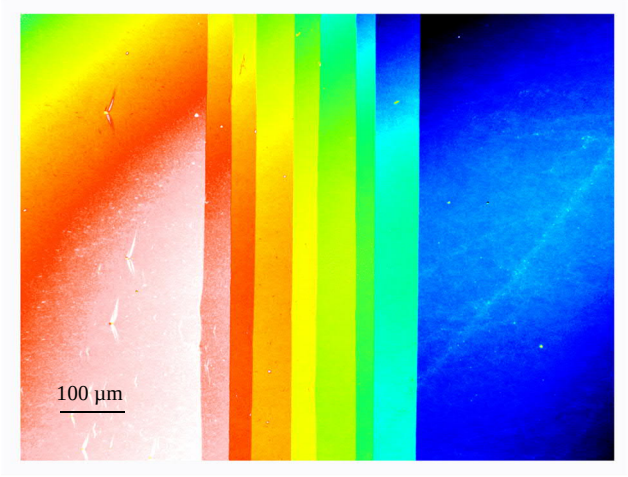

Figure 6. BTS measurement with PSI technique. The color scale has been enhanced to provide a clear view of the residual flatness error between the calibration reference topography and the actual measurement.

To avoid having to calibrate the system and to avoid the influence of the residual errors, a two-measurement subtraction technique was used when measuring the height of the BTS standard. Two profiles are obtained with a lateral shift between them. The left side of Fig. 7 shows two of these profiles obtained with a 20X Mirau interferometer with PSI and 15 pixels apart. Surface tilt and small low frequency components are visible.

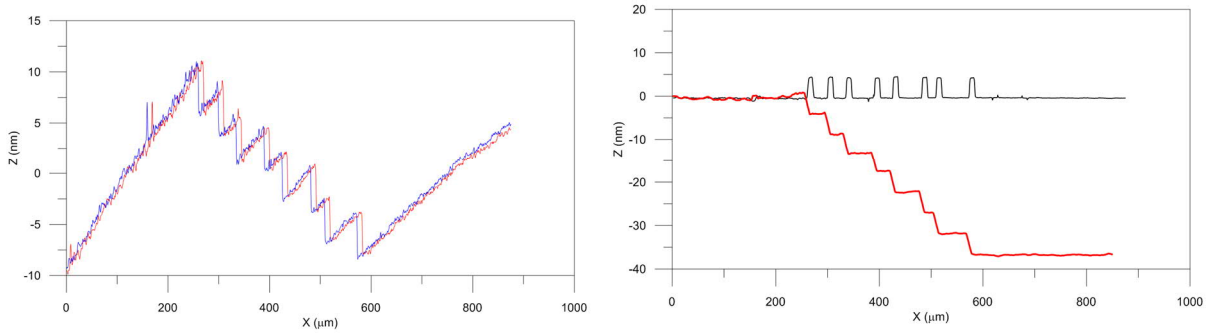

Figure 7. Left: two profiles of a BTS standard taken with a lateral shift of 15 pixels. Right: difference profile (black) and integrated profile (red), which removes the tilt, the calibration mirror topography, and which supresses residual flatness errors.

The right side in Figure 7 shows the difference profile in black, which contains information about the real profile of the sample, while supressing all the effects mentioned above related to the instrument calibration. Each bump corresponds to a step with a width of 15 pixels equal to the profile shift. The red curve shows the accumulation of all points, which corresponds to the real profile of the BTS standard without any calibration influence. This technique supresses the tilt of the surface and any temporal drift due to the fact that both measurements are taken a few seconds apart.

3. RESULTS

The BTS standard manufactured at the University of Helsinki has been measured with two different instruments at two different locations. On each location, two operators measured three BTS specimens; at UH, a custom made white light interferometer in CSI mode was used with a 10X Linnik objective. At Sensofar, the BTS was measured with a S-neox optical profiler in PSI mode and Confocal. For the PSI measurements, two objectives, a 10XDI and 20XDI were used, while for Confocal, a 150X and field stitching was used in order to cover the full field of all individual steps. All measurements were averaged and its standard deviation was calculated. Additionally, each operator with each measurement technique measured the specimen 10 times in order to average any temporal influence on the instrument, such as internal instrument noise, and external environmental influences. Figure 8 shows the BTS standard individual step height values (8 in total) measured in the two locations.

The linearity difference between the two instruments measurements could be explained by several reasons: PSI measurements need to be corrected with the numerical aperture effect, which changes the effectiveness of the wavelength[6]. In the case of the Sensofar's S-neox instrument, the corresponding effective wavelength change was applied using the nominal numerical aperture of the 10XDI and 20XDI objectives, which correspond approximately to 1.022 and 1.050 respectively. On the other side, the wavelength change does not affect to CSI measurements directly, since it does not use the phase information, but it influences on the average scanning speed, and the phase of the interferogram which offsets the envelope position[7]. The phase change due to reflection is dependent on the layer thickness, and thus each individual step will have a different envelope offset. Nevertheless, since the thickness is very small, this effect is constant being at the end nearly negligible for CSI measurements.

Figure 9 shows the standard deviation measured on the two locations for the BTS standard. As can be seen, the standard deviation is very low (close to 0.1 nm) for nearly all the steps for the measurements performed at the University of Helsinki, while the measurements taken at Sensofar have almost a linear relation of step repeatability with step height. The reason for these differences is that all measurements taken at UH were performed with the same measurement technique and

objective, while the measurements taken at Sensofar were performed by using three different objectives and two different measurement techniques.

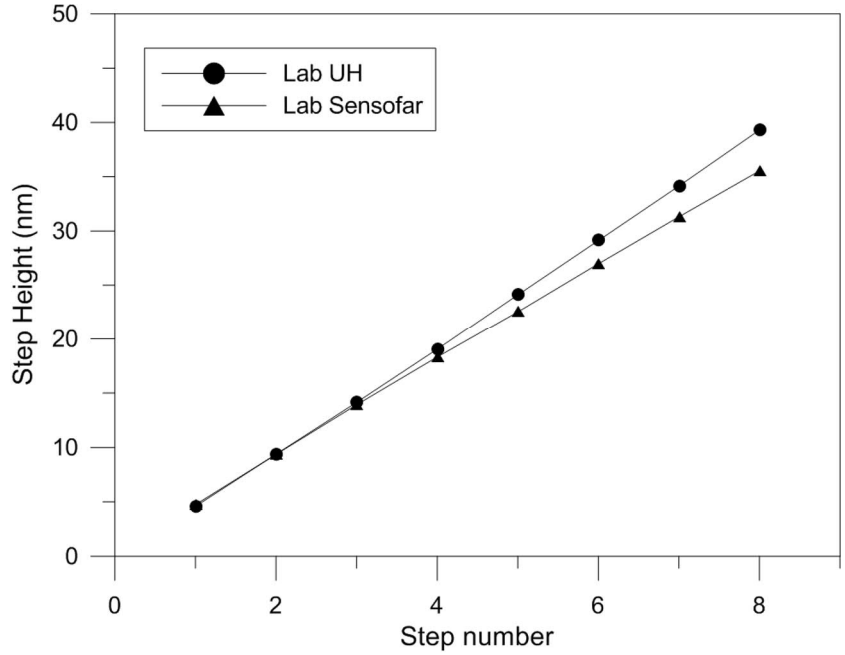

Figure 8. Step height values for the 8 individual steps within the BTS standard on the two measurement locations.

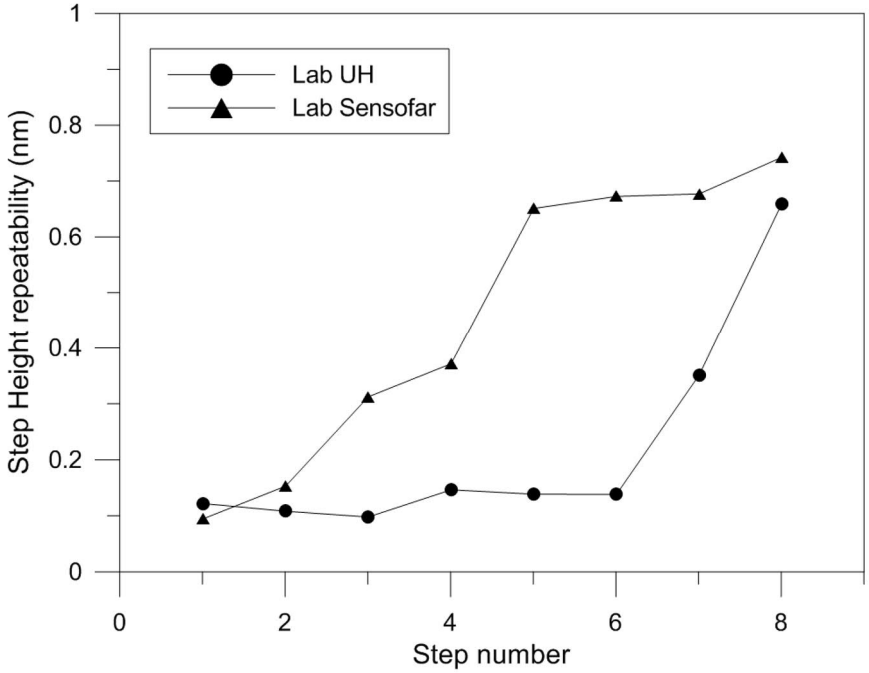

Figure 9. Step height repeatability for the 8 individual steps within the BTS standard measured on the two locations.

4. DISCUSSION

The proposed lateral shift technique used for step height measurements of BTS shows sub-nanometer results for average step height measurements. The results for different users overlap with the combined value and only nanometer-level differences are visible. This is different for the step height repeatability measurements. There are differences between operators in both laboratories, but the common trend is to have bigger standard deviation for bigger step heights.

5. CONCLUSION

The metrology instruments used in both laboratories show good measurement performance with the newly developed transfer standard. The use of self-assembly technique allows step height deviations in the range of ± 0.1 nm in the case of eight step BTS. The proposed number of steps allows using objectives with different magnifications - from 10X up to 150X. The type of the interferometric system Linnik, Michelson or Mirau is not critical for the proposed artefact. This allows the transfer standard to be used also for calibration of other optical or near-field 3D devices.

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